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Role of a putative third subunit YhcB on the assembly and function of cytochrome *bd*-type ubiquinol oxidase from *Escherichia coli*

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Abstract

Recent proteome studies on the *Escherichia coli* membrane proteins suggested that YhcB is a putative third subunit of cytochrome *bd*-type ubiquinol oxidase (CydAB) (F. Stenberg, P. Chovanec, S.L. Maslen, C.V. Robinson, L.L. Ilag, G. von Heijne, D.O. Daley, Protein complexes of the *Escherichia coli* cell envelope. *J. Biol. Chem.* 280 (2005) 34409–34419). We isolated and characterized cytochrome *bd* from the $\Delta yhcB$ strain, and found that the formation of the CydAB heterodimer, the spectroscopic properties of bound hemes, and kinetic parameters for the ubiquinol-1 oxidation were identical to those of cytochrome *bd* from the wild-type strain. Anion-exchange chromatography and SDS-polyacrylamide gel electrophoresis showed that YhcB was not associated with the cytochrome *bd* complex. We concluded that YhcB is dispensable for the assembly and function of cytochrome *bd*. YhcB, which is distributed only in γ -proteobacteria, may be a part of another membrane protein complex or may form a homo multimeric complex.

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1. Introduction

Cytochrome *bd* (CydAB) is one of two terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli*, and predominantly expressed under microaerophilic growth conditions ([1–3]; for reviews). It catalyzes dioxygen reduction with two molecules of ubiquinol-8, leading to the release of four protons from ubiquinols to the periplasm. Through a putative proton channel, four protons used for dioxygen reduction are taken up from the cytoplasm and delivered to the dioxygen reduction site at the periplasmic side of the cytoplasmic membrane [4]. During dioxygen reduction, cytochrome *bd* generates proton-motive force across the membrane through

apparent vectorial translocation of four chemical protons. In contrast to cytochrome *bo* (CyoABCD), an alternative ubiquinol oxidase under highly aerated growth conditions, cytochrome *bd* has no proton pumping activity, and does not belong to the heme-copper terminal oxidase superfamily.

On the basis of spectroscopic and ligand binding studies, three distinct redox metal centers have been identified as heme *b*₅₅₈, heme *b*₅₉₅, and heme *d* (3 for a review). Heme *b*₅₅₈ is a low-spin protoheme IX, and is ligated by His186 (helix V) and Met393 (helix VII) of subunit I (CydA) [5]. Heme *b*₅₉₅ is a high-spin protoheme IX bound to His19 (helix I) of subunit I [5], and mediates electron transfer from heme *b*₅₅₈ to heme *d*, where dioxygen is reduced to water. Heme *d* is a high-spin chlorin bound to an unidentified nitrogenous ligand [6–8], and forms a di-heme binuclear center with heme *b*₅₉₅ [9–11]. Topological analysis suggests that all the hemes are located at the periplasmic end of transmembrane helices [4]. Inhibitor binding studies indicated the close proximity of heme *b*₅₅₈ to the quinol oxidation site [12, 13]. Biochemical [14–16] and mutagenesis [17] studies demonstrated

Abbreviations: PAGE, polyacrylamide gel electrophoresis; DS, dodecyl sucrose

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the presence of the ubiquinol oxidation site in loop VI/VII (Q-loop) of subunit I, that transfers electrons to heme b_{558} bound to the periplasmic ends of helices V and VII.

Cytochrome *bd* is widely distributed among bacteria and archaeobacteria, and is assumed to be a hetero-dimeric oxidase (CydAB, 100,631 Da) [18,19]. However, proteome studies on the *E. coli* membrane proteins using blue-native polyacrylamide gel electrophoresis (PAGE¹), followed by SDS-PAGE, recently postulated YhcB (15,230 Da), an orphan protein, as a putative third subunit [20]. To probe the role of YhcB, we isolated and characterized cytochrome *bd* from the wild-type and $\Delta yhcB$ mutant. We found that YhcB was not associated with the CydAB heterodimer and was dispensable for the assembly and function of cytochrome *bd*. YhcB, which is found only in γ -proteobacteria, may be a part of another membrane protein complex or may form a homo multimeric complex.

2. Materials and methods

2.1. Strains and culture

The *E. coli* strain GR84N/pNG2 (*cydA2 recA/cyd⁺* Tet^R) [21] was a kind gift of R. B. Gennis (Univ. Illinois). pNG2, a pBR322 derivative, carries the *cydAB* operon and can overproduce cytochrome *bd* [22]. The cytochrome *bo*-deficient mutant BL21cyo (BL21 Δcyo , DE3) was constructed as follows. The $\Delta cyo::Km^R$ locus was transduced into BL21 (DE3) by P1 phage grown on strain ST4533 (W3092 $\Delta cyo::kan recA$) [23]. For overproduction of cytochrome *bd*, BL21cyo and a transposon insertion disruptant, JD24286 ($\Delta yhcB::mini-Tn10$ (Km^R); NIG collection), were transformed with pNG2. Cells were grown overnight in IM medium supplemented with trace metals [24], 0.5% glucose, and 12.5 μ g/ml tetracycline. For the culture of BL21cyo, tetracycline was omitted.

2.2. Isolation of cytochrome *bd*

Cytochrome *bd* was solubilized with 0.5 or 2.5% dodecyl sucrose (DS; Mitsubishi-Kagaku Foods Co., Tokyo) from cytoplasmic membranes, and isolated by anion-exchange high performance liquid chromatography on a TSKgel SuperQ-5PW column (21.5 mm I. D. \times 15 cm; Tosoh) [17,25]. Peak fractions were concentrated by ultrafiltration with Amicon Ultra-15 (MWCO 50K), and subjected to gel filtration chromatography with a HiLoad 16/60 Superdex 200 pg column (Amersham) at the flow rate of 1 ml/min in 50 mM sodium phosphate (pH 6.8) containing 0.1% DS, 0.1 mM phenylmethanesulfonyl fluoride (Sigma), and 0.3 M NaCl. Purified enzymes in 50 mM sodium phosphate (pH 6.8) containing 0.1% DS were stored at -80°C until use. Concentrations of protein, heme B and heme D were determined as described previously [17,25].

2.3. Absorption spectroscopy and oxidase assay

Absorption spectra of the air-oxidized and sodium hydrosulfite-reduced forms were determined with a V-550 UV/Vis spectrophotometer (JASCO, Tokyo) at a final concentration of 5 μ M in 50 mM sodium phosphate (pH 7.4) containing 0.1% DS. Enzymes were diluted to 10 nM with 50 mM sodium phosphate (pH 7.4) containing 0.1% DS, and ubiquinol-1 oxidase activity was determined at 25°C [17,25]. Data analysis was carried out with Kaleidagraph ver. 3.5.1 (Synergy Software) by assuming the modified ping-pong bi-bi mechanism [25].

3. Results and Discussion

3.1. Assembly of cytochrome *bd*

YhcB is a monotopic membrane protein with the N-terminus anchor (Fig. 1), and has been identified in cytoplasmic

membranes isolated from the mid-logarithmic growth phase cells [26]. Using the late logarithmic phase cells of the *E. coli* strain BL21 (DE3)/pLysS, Stenberg et al. [20] recently carried out proteome studies on the *E. coli* membrane proteins. Cytoplasmic membrane proteins have been solubilized with 0.5% *n*-dodecyl- β -maltoside, and subjected to two-dimensional blue-native/SDS-PAGE, followed by mass spectrometric analysis. They found that YhcB was associated with cytochrome *bd* as a putative third subunit. However, because of poor resolution of protein spots in two-dimensional gels and a lack of enough molecular weight markers for cytoplasmic membrane proteins (i.e., succinate dehydrogenase (SDH) trimer, cytochrome *bo*, and glucose dehydrogenase (GDH) in supplementary Fig1A of [20]), the assignment of YhcB as the third subunit of cytochrome *bd* remains tentative.

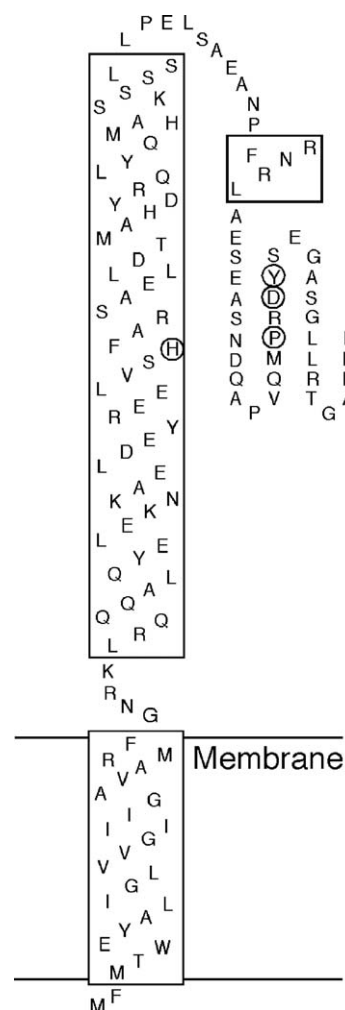


Fig. 1. A topological model for the *E. coli* YhcB. A putative transmembrane helix was determined with SOSUI (available at <http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>), and secondary structures were estimated with PROFsec (available at <http://www.predictprotein.org>). Conserved residues are encircled, and putative helices are indicated by box. PROFsec and others (SSTHREAD and ROBSON; available at <http://mbs.cbrc.jp/papia-cgi>) suggest the presence of a large hydrophilic helix. The alignment of leucine residues suggests protein–protein interactions with a potential coiled-coil structure (e.g., the formation of a homo multimer).

To examine the possible role of YhcB on the assembly and function of cytochrome *bd*, we characterized cytochrome *bd* isolated from the wild-type strains (*yhcB*⁺) and $\Delta yhcB$ mutant. We solubilized cytoplasmic membranes from the cytochrome *bo*-deficient mutant BL21cyo and its cytochrome *bd*-overproducing strain BL21cyo/pNG2 with 0.5% DS, and those from cytochrome *bd*-overproducing strains JD24286 ($\Delta yhcB$)/pNG2 and GR84N (*cydA2*)/pNG2 with 2.5% DS. Elution profiles of cytochrome *bd* on anion-exchange chromatography were identical irrespective of the difference in the detergent concentration and the genetic background (Fig. 2A and data not shown). SDS-PAGE analysis showed that a 15-kDa protein like YhcB was not associated with cytochrome *bd* (CydAB) (Figs. 2B and 3). Our data are consistent with the previous reports, which have shown the heterodimeric organization of cytochrome *bd* [18,19].

Because of binding of detergent molecules to hydrophobic surface of membrane proteins, calibration curves for intrinsic membrane proteins are often deviated from that for soluble proteins (dotted lines in Fig. 4A and B). In gel filtration, apparent molecular mass of the cytochrome *bd* complex was estimated to be ~105 kDa (solid line ($R=0.919$) in Fig. 4A),

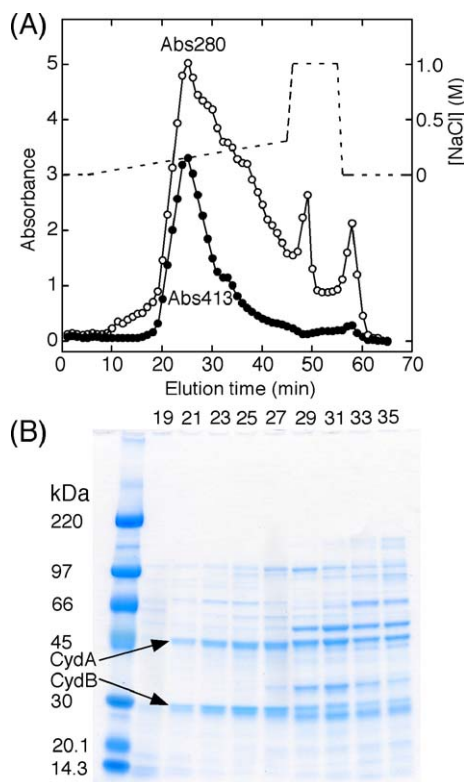


Fig. 2. Elution profile of cytoplasmic membrane proteins from the cytochrome *bo*-deficient mutant BL21cyo on anion-exchange chromatography. (A) High performance liquid chromatography. Cytoplasmic membranes were solubilized with 0.5% dodecyl sucrose, and the supernatant after ultracentrifugation was applied to a TSKgel SuperQ-5PW column. Cytochrome *bd* was eluted with the 0–0.3 M NaCl gradient for 40 min at the flow rate of 5 ml/min in 20 mM sodium phosphate (pH 6.8) containing 0.1% DS and 0.1 mM phenylmethanesulfonyl fluoride. The elution profile was monitored at 280 and 413 nm. (B) SDS-PAGE. Aliquots (2.5 μ l each) around the peak fraction for cytochrome *bd* were subjected to 5–20% SDS-PAGE, and proteins were visualized with GelCode (Pierce).

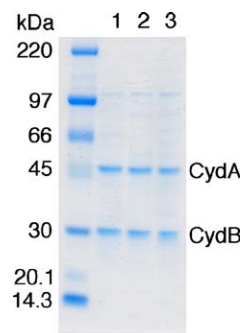


Fig. 3. SDS-PAGE analysis of cytochrome *bd* isolated from BL21cyo (lane 1), BL21cyo/pNG2 (lane 2), and JD24286 ($\Delta yhcB$)/pNG2 (lane 3). The purified cytochrome *bd* (2.5 μ g each) was subjected to 5–20% SDS-PAGE, and proteins were visualized with GelCode.

and is comparable to 100,631 Da expected from the DNA sequence. If we include F_0F_1 -ATPase (554 kDa) and the F_0 subcomplex (176 kDa) to internal standards for the calibration curve of blue-native PAGE (supplementary Fig1A in [20]), we will have the same molecular mass for cytochrome *bd* (~105 kDa (solid line ($R=0.981$) in Fig. 4B) rather than 118 kDa reported by Stenberg et al. [20]. Because of the presence of gel electrophoresis band smiling (i.e., curvature of

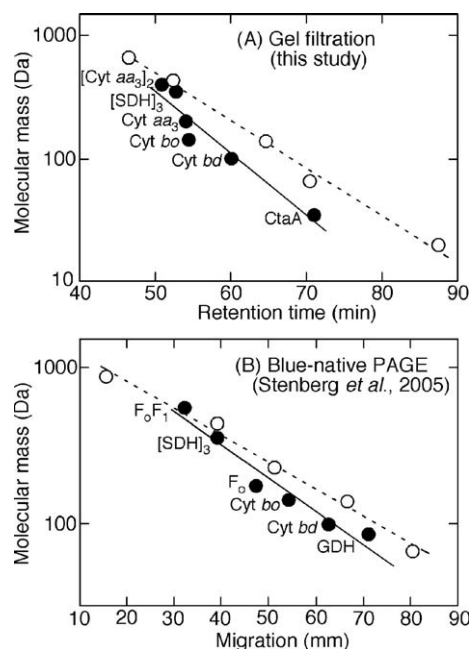


Fig. 4. Estimation of molecular mass of the *E. coli* cytochrome *bd* complex. (A) Gel filtration (this study). Molecular weight markers used are thyroglobin (669 kDa), ferritin (443 kDa), alcohol dehydrogenase (140 kDa), serum albumin (66.3 kDa), and soybean trypsin inhibitor (20.1 kDa) (Daiichi Pure Chemicals, Tokyo). Membrane proteins used are bovine cytochrome *aa*₃ (Cyt *aa*₃; 408 and 204 kDa for a dimer and monomer, respectively), the *E. coli* succinate dehydrogenase trimer ([SDH]₃; 355 kDa), the *E. coli* cytochrome *bo* (Cyt *bo*; 141 kDa), the *E. coli* cytochrome *bd* (Cyt *bd*), and the gene-engineered heme A synthase (CtaA) from *Bacillus subtilis* (35 kDa; details will be described elsewhere). Cytochrome *bo* appears to be a dimer. (B) Blue-native PAGE. Data are taken from Fig. 2 of Stenberg et al. [20]. F_0F_1 , F_0 and GDH indicates F_0F_1 -ATPase, its F_0 subcomplex, and glucose dehydrogenase, respectively.

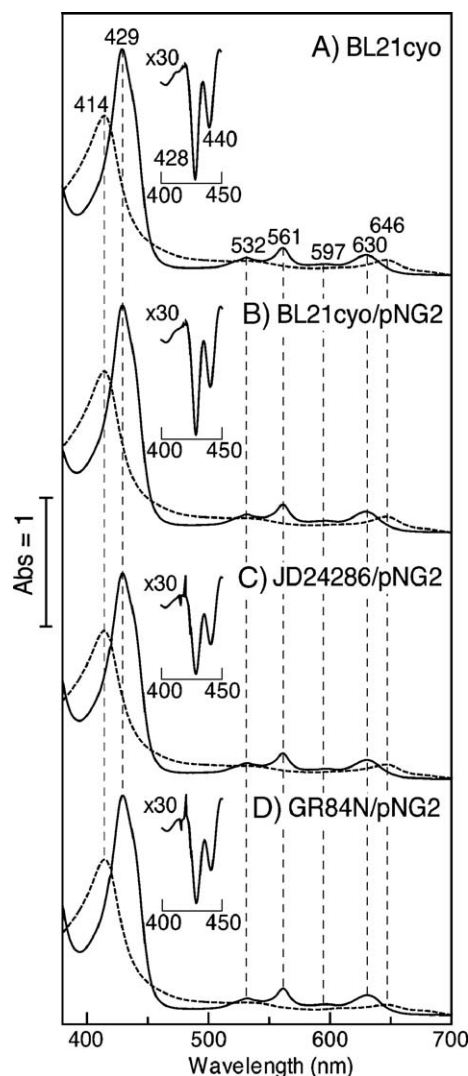


Fig. 5. Absorption spectra of the air-oxidized and fully reduced forms of cytochrome *bd* isolated from (A) BL21cyo, (B) BL21cyo/pNG2, (C) JD24286/pNG2, and (D) GR84N/pNG2. The isolated cytochrome *bd* was diluted to 5 μ M with 50 mM sodium phosphate (pH 7.4) containing 0.1% DS, and absorption spectra of the air-oxidized (broken line) and dithionite-reduced (solid line) forms were measured at room temperature. Inset: the second-order finite difference spectra of the Soret peak, to which heme b_{558} and b_{595} are contributed.

migration bands), the membrane protein complex associated with YhcB appears to be slightly larger than cytochrome *bd* (Figs. 2A and 3B of [20]). These data do not support the proposal [20] that YhcB is associated with the cytochrome *bd* complex.

3.2. Function of cytochrome *bd*

We measured room-temperature absorption spectra of the air-oxidized and fully reduced forms of cytochrome *bd* isolated from the wild-type strains (BL21cyo, BL21cyo/pNG2, and GR84N/pNG2) and $\Delta yhcB$ mutant (JD24286/pNG). All the preparations showed peaks at 414 and 646 (heme d^{2+} -O₂) nm at the air-oxidized state and 428 (b_{558}^{2+}), 440 (b_{595}^{2+}), 532, 561 (b_{558}^{2+}), 597 (b_{595}^{2+}) and 630 (d^{2+}) nm at the fully reduced state (Fig. 5),

indicating that neither the absence of YhcB nor the overproduction of cytochrome *bd* with pNG2 affect the heme binding during the assembly of the enzyme.

The oxidation of ubiquinol-1 by the *E. coli* cytochrome *bd* shows the sigmoidal concentration-dependence on the substrate concentration due to the presence of an inactive conformer and the coupling of the oxidation of two quinol molecules to the four-electron reduction of dioxygen [25,27]. The rate equation for such a modified *ping-pong bi-bi* mechanism can be expressed as follows [25].

$$v = \frac{V_{\max}[S]^2}{K_m^2 + K_m[S] + [S]^2} \quad (1)$$

Apparent K_m and V_{\max} values for cytochrome *bd* isolated from the wild-type (GR84N/pNG2) and the $\Delta yhcB$ mutant (JD24286/pNG2) were determined to be 81 μ M and 438 e⁻/s, respectively, and 94 μ M and 482 e⁻/s, respectively (Fig. 6). Our data show that the absence of YhcB does not affect kinetic parameters for the quinol oxidation. It should be noted that systematic transposon mutagenesis studies on the *E. coli* genes have revealed that *yhcB* is one of non-essential genes in *E. coli* [28]. In conclusion, YhcB is dispensable for the heme binding and the oxidase functions.

3.3. Role of YhcB

Two-dimensional blue-native/SDS-PAGE approach [20] can facilitate studies on the assembly of a large number of membrane protein complexes and the role of orphan proteins. However, estimation of molecular mass for membrane proteins is difficult because of binding of detergent molecules to the hydrophobic surface of proteins and variations in the relative hydrophobic surface.

Characterization of cytochrome *bd* isolated from the wild-type strains ($yhcB^+$) and $\Delta yhcB$ mutant did not support the proposal by Stenberg et al. [20] that YhcB is associated with the cytochrome *bd* complex. The negligence of gel electrophoresis band smiling [20] likely results in the erroneous conclusion on the subunit structure of the cytochrome *bd* complex. YhcB may

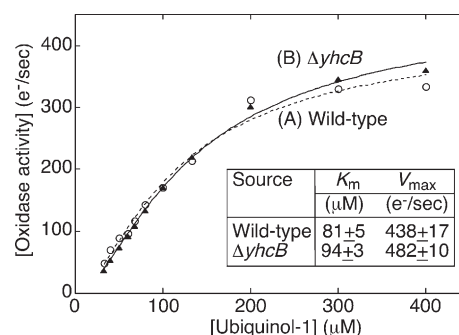


Fig. 6. Kinetic analysis of the ubiquinol-1 oxidase activity of cytochrome *bd* isolated from (A) the wild-type (GR84N/pNG2) (○) and (B) the $\Delta yhcB$ mutant (JD24286/pNG2) (▲). The enzyme concentrations used are 12.5 nM for GR84N/pNG2 and 15 nM for JD24286/pNG2. Inset: kinetic parameters estimated with Kaleidagraph using Eq. (1).

be bound to a membrane complex slightly larger than the CydAB heterodimer or may form a homo multimeric complex.

Our data clearly demonstrate that YhcB is dispensable for the heme binding and the oxidase activity of cytochrome *bd*. The separation of the gene locus of *yhcB* (72.82') from that of *cydAB* (16.6') and the presence of YhcB only in γ -proteobacteria suggest that YhcB has not coevolved with cytochrome *bd*, which is widely distributed among bacteria and archaeobacteria. The role of YhcB remains to be examined but is unlikely related to housekeeping functions (e.g., generation of proton-motive force by cytochrome *bd*). Membrane protein complexes play pivotal roles in energy and signal transduction. Proteome analyses will provide a large volume of information for thousands of proteins instantly. The present study suggests that results obtained should be further examined by genetic and biochemical approaches.

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